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*Louisiana State University and Agricultural and Mechanical College, [ywang11@lsu.edu](mailto:ywang11@lsu.edu)*

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# EVALUATION OF LUTEIN AND PROTEIN IN OZONE TREATED CORN

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

In

The Department of Food Science

By

Yu Wang

B.S., Hefei University of Technology, China, 2003

August 2005

## **Dedication**

I would like to dedicate my research to my grandparents, Mr. Wang, Zhiyong and Mrs. Jin, Shuhe, who have always supported me. Thank you for everything you have done for me. I will treasure them in my whole life.

## **ACKNOWLEDGMENTS**

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## ABSTRACT

The present study was conducted to determine the influence of an ozonation process on lutein and protein in clean and contaminated corns. This study aimed (1) to determine the levels of antimutagenic compounds to aflatoxin in corn before and after ozonation; (2) to verify the antimutagenic potential of these compounds against aflatoxin using the Ames test; and (3) to evaluate proteins in the corn before and after ozonation.

HPLC was used to separate and identify the lutein in corn. Nitrogen analysis and SDS-PAGE were used to analyze protein content and molecular weights in corn. Clean ozone-treated corn had a total lutein content of 28.36 ug/g, which was higher than that of 22.75 ug/g in the untreated clean corn. However, the results of contaminated corn were contrary to clean corn. Lutein content of 11.69 ug/g in the ozone-treated contaminated corn was lower than that of 16.42 ug/g in the untreated contaminated corn. Through the nitrogen analysis, protein content was determined. In both corn samples, the protein content of ozone-treated corn was lower than that of untreated corn, indicating that protein could be destroyed by the ozonation process.

The Ames test using the Salmonella plate incorporation method was used to examine the effect of lutein extracts from corn on aflatoxin B1 mutagenicity, using TA100 tester strain. Lutein extracts alone showed no mutagenic potential against *Samonella typhimurium* tester strains TA 100. The dose of 500 ng AFB1/g was chosen for the antimutagenicity studies. Pure lutein and lutein extracts from corn inhibited the mutagenicity of AFB1 in a dose-response manner. Lutein extracts were more efficient at

inhibiting AFB1 mutagenicity than lutein standard. Lutein extracts from different corn samples had similar antimutagenic potential against AFB1, so the ozone treatment could not change antimutagenic potential of lutein extracts.

Ozonation releases lutein from clean corn, while in the contaminated corn, ozonation destroys some of lutein in the corn. However, ozonation dose not change the antimutagenic potential of lutein against AFB1. Protein can be destroyed by ozone, which influences the nutritious value of corn.

## CHAPTER 1. INTRODUCTION

Aflatoxins are a group of closely related bis-dihydrofurano secondary fungal metabolites which have been implicated as carcinogens in humans. There is a non-specific environment for fungal growth and toxin formation so there are diverse types of foodstuffs contaminated with aflatoxins. The aflatoxin that has caused the most concern is AFB1. Aflatoxin B1 (AFB1) is a potent mutagenic and carcinogenic mycotoxin produced by *Aspergillus flavus* and *A parasiticus* and is frequently found in foodstuffs such as corn, peanuts and cottonseeds (Hsieh 1986). Exposure to aflatoxin B1 is generally considered a major factor in the high incidence of hepatocellular carcinoma, a malignant neoplasm of hepatic cells, commonly referred to as primary liver cancer. It is estimated that as much as one-quarter of the world's yearly food and feed crops are contaminated with mycotoxin (FAO, 1996). The Council for Agricultural Science and Technology (1989) estimated that in the United States alone, twenty million dollars is lost annually on peanuts contaminated with aflatoxin. Hepatotoxic disease is a serious problem, and ultimately leads to the recognition that aflatoxin is both an economic and a public health problem in many areas of the world.

In an effort to limit human exposure to these toxins, prevention and control programs are continuously being studied and established. These include monitoring of commodities susceptible to aflatoxin contamination, the establishment of limits and regulations that are legally enforced, and decontamination procedures designed to remove or inactivate the toxicant in food or feed (Park, 1993). Methods to decontaminate aflatoxin-affected foods

and feed are being studied and evaluated to optimize them or to obtain more efficient and safer methods. Some of the methods include physical, chemical or biological removal, or use of chemical or physical inactivation. The chemical treatments to decontaminate aflatoxin-containing commodities are currently the most practical approach. Although these chemical treatments are effective, they can cause some damage to nutrients, odor, flavor, color and so on. One method of decontamination for aflatoxin-affected foodstuff that has been a focus of attention is ozonation, which was shown to be an effective way to reduce the aflatoxin levels as much as 95% (Prudente and King, 2002; McKenzie, 1997). However, there are some kinds of compounds, which are naturally antimutagenic to the aflatoxin. They are present in the foodstuff to prevent the mutagenicity caused by aflatoxin. So the next step of our research was to find these antimutagenic compounds and detect the levels of these antimutagenic compounds before and after ozonation. Meanwhile, there are some nutrients such as protein, which must also be analyzed after the ozone process. These aspects are very important in assessing the suitability and acceptability of ozonation .

Therefore, this study primarily aimed (1) to determine the levels of antimutagenic compounds in corn before and after ozonation; (2) to verify the antimutagenic potential of these compounds against aflatoxin using the Ames test; and (3) the evaluation of proteins in the corn before and after ozonation.

## **CHAPTER 2. LITERATURE REVIEW**

### **2.1 Aflatoxin**

#### **2.1.1. History**

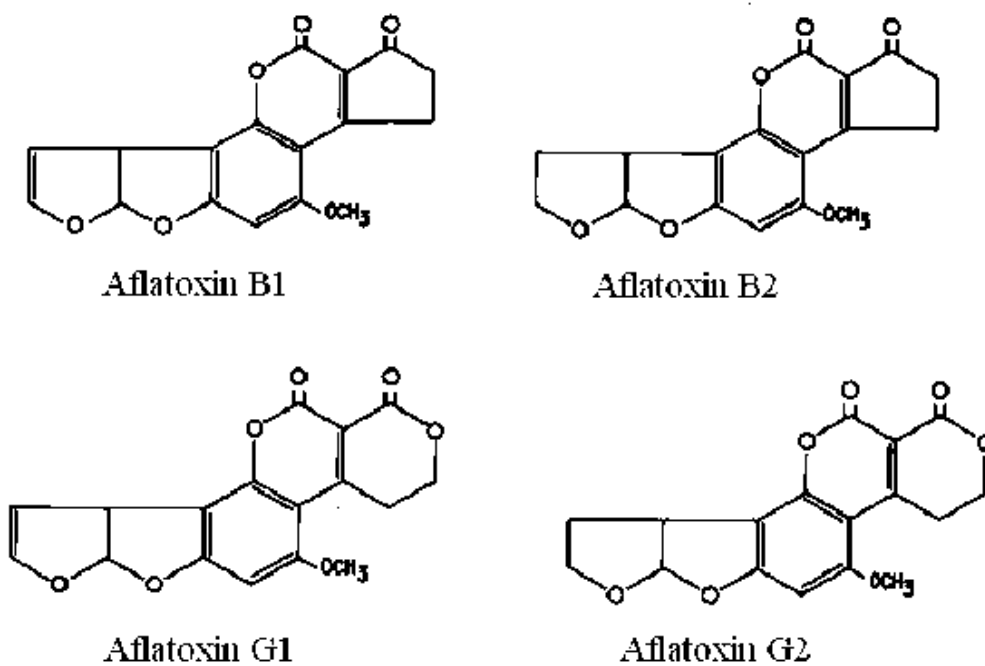
In 1960, there was an acute hepatotoxic disease in turkeys affecting animals in many areas of the world. Then the reports of turkey “X” disease were made of poisoned chickens and ducklings as well as turkeys. These animals were all characterized by acute hepatic necrosis, marked bile duct hyperplasia, acute loss of appetite, wing weakness, and lethargy (Blount, 1961). In fact, in the mid-1940s, Bruce and Parkes found that a diet for laboratory animals that contained peanut meal as a source of part of the protein could cause liver cancer. Furthermore, Paterson et al. (1962) demonstrated that the liver disease was attributed to toxic peanuts in the diet with new research on swine and cattle. The feeds, especially the moldy feeds, could cause the disease. The moldy feeds were contaminated with aflatoxin (Newberne et al., 1966). Meanwhile, the southeast Missouri state laboratories found that trout hepatoma occurred in hatchery-raised fish. The fish were fed a commercial chow that contained cottonseed meal as part of the protein source. Later, this meal was shown to be contaminated with aflatoxins. Hepatotoxic disease is a serious problem, and ultimately leads to the recognition that aflatoxin is both an economic and a public health problem in many areas of the world.

### **2.1.2 Occurrence**

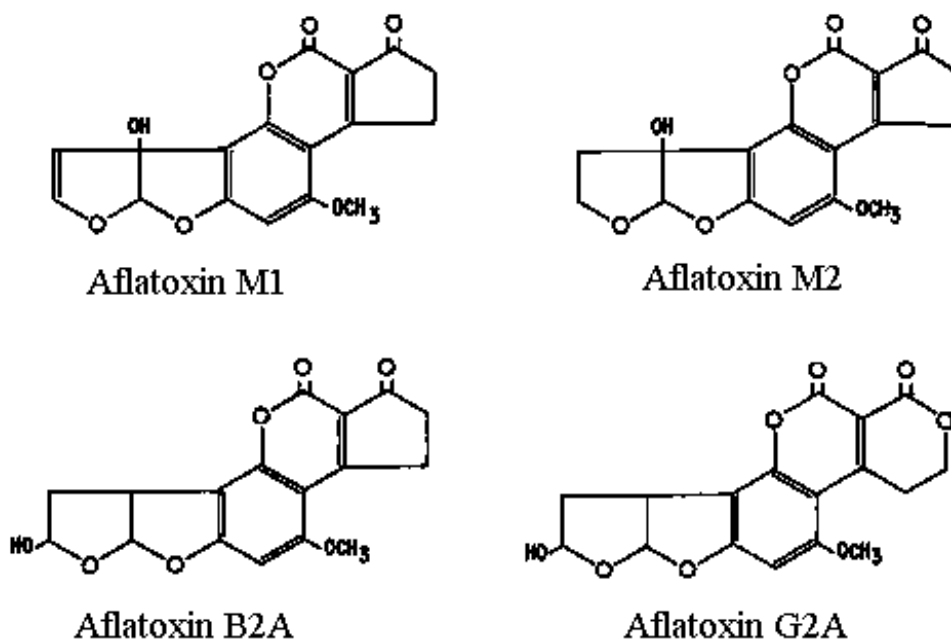
There is a non-specific environment for fungal growth and toxin formation so there are diverse types of foodstuffs contaminated with aflatoxins. For example, cereal (maize, sorghum, pearl millet, rice, wheat), oilseeds (groundnut, soybean, sunflower, cotton), spices (chilies, black pepper, coriander, turmeric, zinger), tree nuts (almonds, pistachio, walnuts, coconut) and milk (Reddy et al., 1995). Aflatoxin can occur in the field prior to harvest and postharvest if the weather is humid, or crop drying is delayed. Milk, eggs and meat products usually are contaminated because the animals are fed with aflatoxin-contaminated food.

### **2.1.3 Physical and Chemical Characteristics**

Aflatoxins are probably the best known and most intensively researched mycotoxins in the world. They are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, and produced as secondary metabolites by the fungus *Aspergillus flavus* and *A parasiticus* on variety of food products. The word aflatoxin is formed from the following set-up: the first letter “A” for the genus *Aspergillus*, the next set of three letters, “FLA” for the species *flavus*, and “TOXIN” for the meaning of poison. (Ellis et al, 1991). According to Reddy et al (1995), there are 18 different types of aflatoxins of which the major forms are aflatoxin B1, B2, G1, and G2 (Figure 2.1). There are four other aflatoxins M1, M2, B2A, G2A (Figure 2.2), which are produced in minor amounts. M1 and M2 are major metabolites of aflatoxin B1 and B2 respectively and are found in milk of animals that consumed feed contaminated with aflatoxins.



**Figure 2.1** The structures of aflatoxins B1, B2, G1, and G2



**Figure 2.2** The structures of aflatoxin M1, M2, B2A, G2A

According to the chemical structure of aflatoxins, they are classified in two groups: difurocoumarocyclo pentenone series (AFB1, AFB2, AFB2A, AFM1, AFM2 and AFM2A), and difurocoumarolactone series (AFG1, AFG2, AFG2A, AFGM1, AFGM2, and AFGM2A). The structures above are B designation and G designation. Under UV-light, the B form exhibits blue fluorescence, while the G designation refers to the yellow-green fluorescence. The aflatoxins display potency of toxicity, carcinogenicity, mutagenicity in the order of AFB1 > AFG1 > AFB2 > AFG2 as illustrated by their LD50 values for day-old ducklings (Reddy et al., 1995).

**Table 2.1** Chemical and physical properties of aflatoxins

Aflatoxin	Molecular formula	Molecular weight	Melting point
B <sub>1</sub>	C <sub>17</sub> H <sub>12</sub> O <sub>6</sub>	312	268-269
B <sub>2</sub>	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>	314	286-289
G <sub>1</sub>	C <sub>17</sub> H <sub>12</sub> O <sub>7</sub>	328	244-246
G <sub>2</sub>	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>	330	237-240
M <sub>1</sub>	C <sub>17</sub> H <sub>12</sub> O <sub>7</sub>	328	299
M <sub>2</sub>	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>	330	293
B <sub>2A</sub>	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>	330	240
G <sub>2A</sub>	C <sub>17</sub> H <sub>14</sub> O <sub>8</sub>	346	190



## **2.1.4 Metabolism**

### **2.1.4.1 Absorption and Distribution**

Most of the pharmacokinetic considerations are associated with the administration of relatively low doses of AFB1 since the typical route for human exposure to aflatoxins occurs mainly through ingestion of contaminated foodstuffs (Hsieh and Wong, 1994). It is efficient for AFB1 to be absorbed after ingestion because of its characteristics of being a low molecular weight lipophilic compound (Wogan, 1996).

In absorption studies, Kumarann (1989) showed that AFB1 could be rapidly absorbed from intestines into the mesenteric venous blood. The duodenum was considered the site of most efficient absorption. The author also reported that the degree of aflatoxin uptake was proportional to AFB1 concentration which indicated that it is absorbed by passive diffusion.

From the intestine, AFB1 apparently enters the liver, where the toxin can be concentrated and metabolized, through the hepatic portal blood supply (Wilson, 1985). According to the Hsieh and Wong's Study (1994), the kidney has been shown to concentrate aflatoxin to a lesser extent.

### **2.1.4.2 Phase I Metabolism**

The most potent of the aflatoxins is considered to be AFB1 which is related to benzofurans in most genotoxicity assays and is the most hepato-carcinogenic (Busby and Wogan, 1984). However, aflatoxin B1 by itself is not particularly genotoxic. Most of the mutagenic and toxic properties of aflatoxin B1 are attributed to its reactive metabolite,

the exo-8, 9-epoxide. It is produced via oxidation by cytochrome P450 3A4 and cytochrome P450 2A5 which are predominant catalysts in the human and mouse family, respectively (Pelkonen et al., 1997).

Cytochrome P450 enzymes are involved in the oxidation of numerous steroids, eicosanoids, alkaloids, and other endogenous substrates. These enzymes are also involved in the oxidation of potential toxicants and carcinogens such as those encountered among environmental pollutants, solvents, pesticides, and many natural toxins like aflatoxins (Guengerich et al., 1998). P450 enzymes produce two different stereometric forms of the 8,9 epoxide, the exo-8,9 epoxide and endo-8,9 epoxide. The exo isomer is at least 1000 times more genotoxic than its endo counterpart (Guengerich et al., 1996). The exo isomer of the epoxide is considered a strong electrophile that can form covalent adducts with macromolecules such as proteins, RNA and the N-7 position of guanine residues in DNA (Foster et al., 1983; Miller, 1991).

Cytochrome P450 isozymes produce a number of products that can be considered activated metabolites (i.e, 8,9 epoxide) or detoxification products such as aflatoxins M1, P1, and Q1 which do not interact with DNA (Langouet et al., 1996). Among the P450s, CYP3A4, CYP1A2, CYP1A1 and CYP3A5 have been shown to transform AFB1. CYP1A2 readily oxidizes AFB1 to AFM1, AFQ1 and AFB1 endo-8,9-epoxide. CYP3A4 oxidizes AFB1 to AFQ1, as well as the exo epoxide (Guengerich et al., 1998).

#### **2.1.4.3 Phase II Metabolism**

In Phase II metabolism, the metabolites in Phase I can be conjugated to less toxic compounds which are more available for excretion. The major Phase II metabolites are the glutathione, glucuronide and sulfate conjugates (Hsieh and Wong, 1994). Glutathione (GSH) conjugate of the aflatoxin exo-8,9-epoxide has been identified as the major Phase II reaction. Glucuronide conjugation also plays a role in biotransformation and excretion of AFB1 and/or its metabolites. In trout, glucuronides of aflatoxicol and aflatoxin-M are the principal biliary metabolites (Loveland et al., 1984). Phase II metabolites such as aflatoxins P1, Q1 and M1 go through glucuronide conjugation. Metcalfe and Neal (1983) reported that the rate of conjugation of these metabolites differs and the phenolic hydroxyl group present in AFP1 is a much better site for glucuronide conjugation than the hydroxyl groups present in AFM1 and AFQ1.

#### **2.1.4.4 Competitive Pathway: Activation and Detoxification**

The toxicity of AFB1 is dependent on a delicate equilibrium between several pathways. The amount of aflatoxin available to exert toxic, mutagenic and carcinogenic effects will depend on the amount converted to the various metabolites and their relative biological activity. The hydroxylated compounds of aflatoxin B1 such as AFM1, AFP1 and AFQ1 are considered detoxification products because of their lower ability to react with DNA and proteins (Eaton et al., 1994; Syed, 1999). AFB1 exo-8,9-epoxide is the essential active metabolite with respect to carcinogenicity. However, the 8,9 epoxide is not always a toxic product since reactive detoxification of this molecule may occur

through phase II conjugation with glutathione. Hydrolysis of the electrophilic epoxide to form a dihydrodiol also represents a decrease in toxic potential. The hydrodiol can exist in a resonance form as a phenolate ion which is capable of forming Schiff's base adducts with a protein amino group, particularly lysine (Lin et al., 1978).

It has been noted that humans and animals are chronically exposed to low doses rather than to one acute single dose. Furthermore, in most in vitro studies, AFB1 concentrations surpass the actual dietary human exposure. Thus, it is difficult to extrapolate from in vitro studies. According to Ramsdell and Eaton (1990), the activity of the different metabolizing enzymes varies from species to species. Moreover, affinity to detoxification reactions also plays an important role in AFB1's ultimate toxic effect. The affinity of the enzyme glutathione S-transferase to the AFB1-8,9-epoxide varies across species.

In different species, the activation and inactivation ratios are a convenient way of comparing the relative activities of AFB1 biotransformation pathways (Degen and Neumann, 1998). They defined activation as the amount of AFB1-8,9-epoxide formed divided by the total amount of oxidative metabolites (AFM1+AFP1+AFQ1) and inactivation is the amount of epoxide conjugated with glutathione divided by the amount of epoxide formed. An index of species susceptibility can be calculated as activation/inactivation.

#### **2.1.4.5 Toxicity**

The aflatoxin that has caused the most concern is AFB1 due to its widespread occurrence, its prevalence among the four naturally occurring aflatoxins, and its acute toxicity and carcinogenicity (McKenzie, 1997). The liver is considered the primary target organ for aflatoxin toxicity. Acute aflatoxicosis has been characterized by vomiting, abdominal pain, pulmonary edema, fatty infiltration and necrosis of the liver (Shank, 1981). There is ample evidence for substantial human exposure in certain populations, but information on clinical aflatoxicosis in humans is still limited (Busby and Wogan, 1984).

The acute toxicity of the aflatoxins is the carcinogenic potential of AFB1 (Wogan et al., 1971; McKenzie, 1997). In the short term study, the results of a single large or repeated dose of aflatoxins include hepatocyte regeneration, bile duct proliferation, and fibrosis, while in the long term study, hepatocarcinoma or occasionally, renal, colon, or other carcinomas are developed (Newberne and Rogers, 1981). Aflatoxin carcinogenicity has been studied intensively and has been demonstrated on poultry or rainbow trout (Hendricks, 1994) and rats (Roebuck et al., 1991). Aflatoxins may be carcinogenic to man in epidemiological studies and there are reports of cases of primary liver cancer (PLC) in primates (Ellis et al., 1991). Although a direct effect relationship has not been confirmed, the association between mycotoxin exposure and PLC is suggested by correlation of exposure to aflatoxins and PLC incidence rates in some areas of Africa and Asia (Shank et al., 1972; Peers and Linsell, 1973; Peers et al., 1976; Van Rensberg et al.,

1985; Hsieh, 1986; Peers et al., 1987; Groopman et al., 1988; Yeh et al., 1989). In 1987, the International Agency for Research on Cancer (IARC) concluded that there was sufficient evidence to classify aflatoxin as a human carcinogen.

## **2.2 Detoxification/ Decontamination Procedures for Aflatoxin**

Practical and economical detoxification procedures are needed for the presence of mycotoxins in foods and feeds. Such methods should not only reduce the concentration of toxins to safe levels but they should also not produce toxic degradation products nor reduce the nutritional value of the treated commodity. A number of approaches have been taken to detoxify mycotoxins; however, only a few have apparent practical applications. The means of degrading mycotoxins will be grouped into three categories: a) physical; b) chemical; and c) biological.

### **2.2.1 Physical Methods**

Aflatoxin is quite stable to heat. The degradation of aflatoxin AFB<sub>1</sub> in peanut and corn oil is at the temperature of 250°C (Peers and Linsell, 1975). The moisture content of the heated product is a critical factor in degrading aflatoxin. Mann et al. (1967) reported that increasing the moisture content of oil seed meal resulted in elevated rates of aflatoxin degradation. In their studies, 85% of the toxin was degraded in the meal containing 30% moisture as compared to 30% degradation in the meal containing less than 7% moisture. Generally, temperatures higher than 100°C are required for partial degradation of aflatoxin, but these processes are not used due to the possibility of generating toxic pyrolysates in food and degrading other nutrients.

There is no effectiveness of gamma and UV irradiation on the decontamination of commodities (Feuell, 1966; Frank and Grunewald, 1970). In their studies, the level of exposure to x-rays, gamma rays and electron irradiation required to destroy aflatoxins would also destroy the irradiated commodity. Furthermore, the complex organic substances, such as aflatoxins are seldom attacked by gamma rays directly. In water or other simple compounds, an indirect effect may produce reactive free radicals that can lead to aflatoxin degradation. The production of toxic degradation compounds of AFB1 constitutes another important limitation for using irradiation (Samarajeewa et al., 1990).

The role of absorption has been reported as an effective means of decontaminating solutions in the elimination of aflatoxin from the contaminated media. Bentonite clay can be used in the removal of aflatoxin from the liquid mediums such as milk (Masimango et al., 1978). The ability of bentonite to adsorb and retain aflatoxin is dependent on the particle size and the degree of heat treatment. Some studies have been done on other substances such as alumina, silica, and aluminosilicates, which were based on their adsorptive properties. In these substances, hydrated sodium calcium aluminosilicate has been found to have a high affinity to AFB1 through formation of a stable complex with AFB1 (Ellis et al., 1991).

### **2.2.2 Chemical Methods**

A variety of chemicals capable of degrading aflatoxins have been studied extensively. Although many chemicals can destroy aflatoxin, the number that can do so without leaving deleterious residues or without excessive damage to nutrients appear to be small.

In food production, it is essential to consider that the nutrient retention, odor, flavor, texture, and functional properties can be accepted by the consumers (Goldblatt and Dollear, 1977).

Chemicals such as chlorinating compounds (chlorine dioxide, sodium hypochlorite) and oxidizing agents (hydrogen peroxide, ozone, and sodium bisulfite) have been evaluated for their detoxifying properties (Samarajeewa et al., 1990; Park et al., 1981). Methods for the use of chemicals including calcium hydroxide (Park et al., 1981), ammoniation of contaminated corn (Park et al., 1988), nixtamalization in corn tortillas (Price and Jorgensen, 1985), and modified nixtamalization (calcium hydroxide + hydrogen peroxide and sodium carbonate) (Lopez-Garcia, 1995), have been developed. The relative efficiencies of various alkalis to destroy AFB1 in liquid media at 110°C are: ammonium carbonate < sodium bicarbonate < ammonium hydroxide < potassium bicarbonate < sodium carbonate < potassium carbonate < sodium hydroxide < potassium hydroxide.

Bisulfite, which inhibits both enzymatic and non-enzymatic browning, acts as an antioxidant and as a reducing agent, and retards the growth of microorganisms, is an acceptable and commonly used food additive in beverages, fruits, and vegetables (Robert and Mc Weeny, 1972; Doyle and Marth, 1978a; Doyle and Marth, 1978b). Meanwhile, bisulfite can degrade aflatoxins B1 and G1. Fifty percent of aflatoxin B1 and G1 could be degraded at 25°C by using an aqueous solution with a pH of 5.5. Moreover, the higher the temperature, the higher rate of aflatoxin degradation (Doyle and Marth, 1978a; Doyle



and Marth, 1978b). However, more studies are needed to demonstrate the safer use of sulfites in detoxification.

Ammoniation, which is an effective and economically feasible means for reducing the aflatoxin from food and feed stuff, is one of the alkali treatments that have been most intensively studied (Park et al., 1988). The ammoniation process, using either ammonium hydroxide or gaseous ammonia, has been shown to reduce aflatoxin levels by more than 99% (Park and Liang, 1993). Both have no effect on non-protein nitrogen content in peanut and cottonseed (Park et al., 1988). The process is irreversible if it proceeds sufficiently (Weng et al., 1994). The use of ozone as a decontamination agent will be discussed in a separate section.

### **2.2.3 Biological Methods**

The biological detoxification of aflatoxin-contaminated commodities involves the use of microorganisms such as bacteria, actinomyces, yeasts, molds, and algae, which have the capability to degrade AFB1 to less toxic compounds. Bacterium, *Flavobacterium aurantiacum* NRRL B-184, is able to remove aflatoxin completely from contaminated milk, corn oil, peanut butter, and corn, and partially detoxify contaminated soybean (Ciegler et al., 1966). Other microorganisms were also found to be capable of converting or transforming AFB1 to aflatoxicol that is about 18 times less toxic than AFB1. However, the conversion is a very slow and incomplete process and can take 3 to 4 days (Detroy and Hesseltine, 1969).

Molds that are capable of producing aflatoxins may also degrade them. In a study by Ellis et al (1991), the production of aflatoxin by *A. parasiticus* and *A. flavus* reached a maximum and then decreased during continuous incubation of the culture. It is thought that these degradation reactions occur through enzymatic activity and these enzymes produce products or by products that react with aflatoxin.

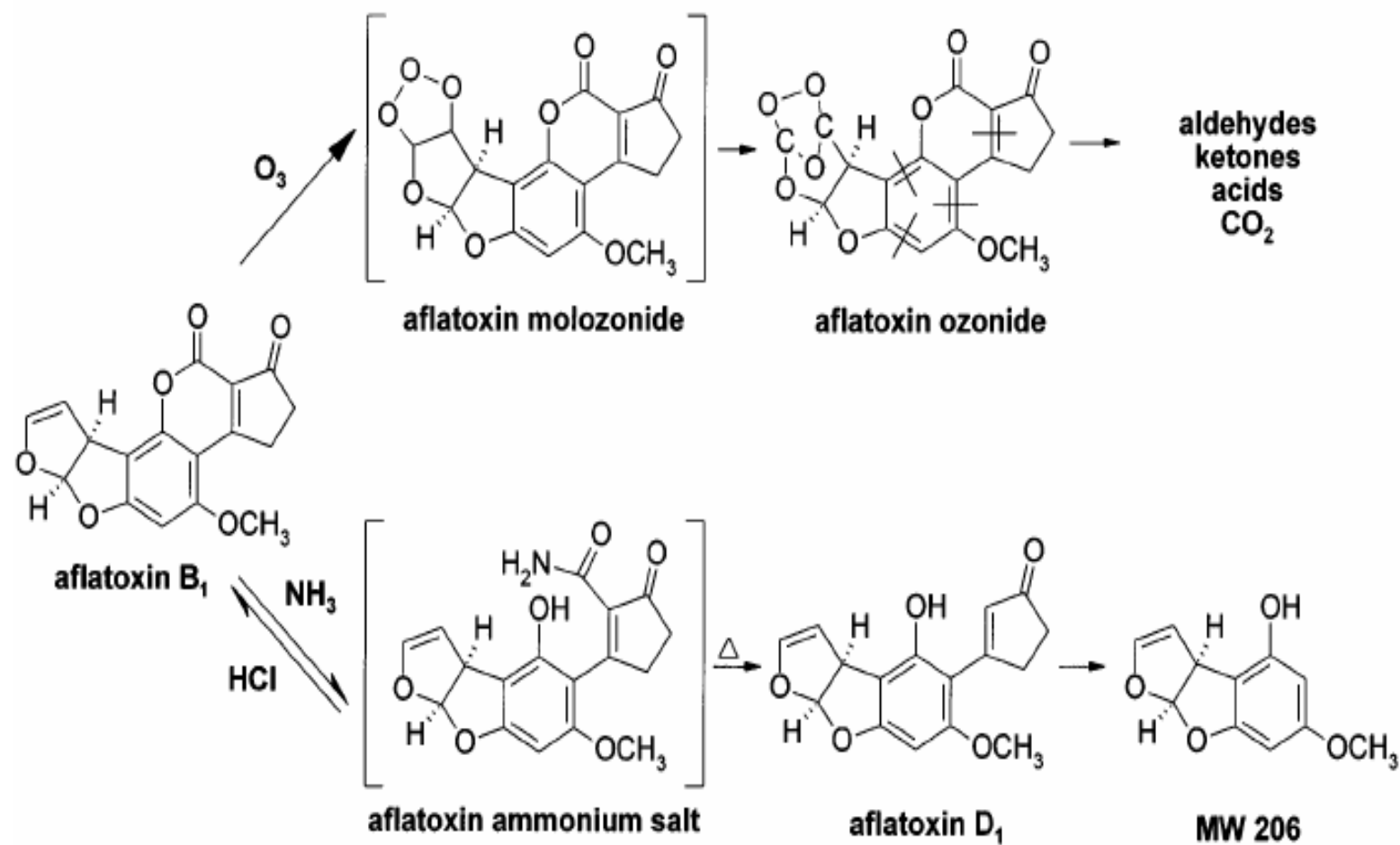
Nontoxigenic strains of *A. parasiticus* and *A. flavus* fungi can be used in the prevention of aflatoxin contamination of peanuts and cottonseeds pre-harvest. Because they can compete with and exclude toxin producing strains (Phillips et al., 1994). Aflatoxin contamination in the peanut can be reduced over a 3-year period because of application of competitive, nonaflatoxigenic strains of *A. parasiticus* (Dorner et al., 1992). Dorner et al. (1999) indicated that a combination of nonaflatoxigenic strains of *A. flavus* and *A. parasiticus* used in soil resulted in a reduction in aflatoxin contamination of peanuts from 74.3 to 99.9%.

#### **2.2.4 Degradation of Aflatoxin by Ozonation**

Triatomic oxygen or ozone is a bluish gas with a pungent and characteristic odor. It has a molecular weight of 48, boiling point of  $-111.9^{\circ}\text{C}$ , and melting point of  $-192.7^{\circ}\text{C}$  (Merck index, 1989). Ozone weighs approximately 0.135lb/cu. ft. and its oxidation potential is high (-2.07v) compared to that of hydrochloric acid (-1.49v).

Research had been done to evaluate the effects of ozone gas in reducing aflatoxin concentration in aflatoxin contaminated agricultural products. Dwarakanath et al. (1968) reported that ozone (25 mg/min) reduced aflatoxins in cottonseed meal and peanut meal.

In cottonseed meal, 91% of the total aflatoxin was destroyed and decreased from 214 to 20 ppb in 2 hours. In peanut meal, 78% of aflatoxin was destroyed from 82 to 18 ppb in 1 hour. Another similar study on peanut meal showed that ozone (25mg/min) could inactivate aflatoxin as indicated by TLC analysis and feeding experiments with ducklings and rats (Dollear et al., 1968). In 1988, Maeba and coworkers showed the inactivation of pure aflatoxins by ozone (1.1mg/l, 5min) in a model system and subsequent reduction of mutagenic activities in the Ames assay. In 1997, McKenzie developed a novel and continuous source of ozone gas through electrolysis. He treated corn spiked with aflatoxins and naturally contaminated rice powder with ozone. He reported a rapid degradation of AFB1 and AFG1 using 2 wt.% ozone, while AFB2 and AFG2 were more resistant to oxidation and needed higher levels of ozone. In a similar study in 1998, McKenzie indicated that aflatoxins could be reduced by 95% in samples treated with 14 wt% ozone for 92 hours at a flow rate of 200 mg/min. Prudente and King (2002) found that aflatoxin could be reduced 95% by ozone treatment. Turkey poult fed with ozone-treated contaminated corn did not show harmful effects as compared to turkey poult fed with untreated contaminated corn (McKenzie et al., 1998). In their study, they also showed the proposed mechanism for aflatoxin degradation by ozone and ammonia (Figure 2.3).



**Figure 2.3** Degradation of aflatoxin B<sub>1</sub> by ozone and ammonia. The primary site of attack of ozone is at the C8–C9 double bond on the terminal furan; whereas, ammonia opens the lactone ring in the coumarin and leaves the C8–C9 double bond intact. This site on the molecule has been shown to be responsible for aflatoxin’s mutagenicity and carcinogenicity (McKenzie et al., 1998)

### **2.3 Antimutagens**

Foods contain mutagens and carcinogens, some of which occur naturally and others could be introduced during the preparation of foods for consumption. Most of these mutagens are metabolized by cytochrome P450 enzymes, resulting in the formation of reactive compounds to produce DNA adducts. Meanwhile, the human diet contains a number of antimutagens and anticarcinogens (Stich, 1991). The role of dietary factors in the prevention of major chronic diseases, cancer in particular, is under intensive investigation by many researchers around world. However, at present it is difficult to comprehend the role of the antimutagens and anticarcinogens in foods in terms of reducing cancer incidence (Syed, 1999).

Some micronutrient and nonnutrient substances that occur in foodstuffs and can counteract mutagenic and carcinogenic processes could be exploited to benefit both man and livestock. For instance, fruits and vegetables contain antimutagenic and anticarcinogenic compounds, particularly the flavonoids (Sugimura et al., 1996; Tanaka et al., 1997). Chlorophyllin, a food grade derivative of chlorophyll, the ubiquitous pigment in green plants, has been shown to be a potent dose-reponse inhibitor of the mutagenicity of several compounds including benzo[a]pyrene, 2-aminoanthracene and aflatoxin B1 by preventing DNA adduction and hepatocarcinogenesis in rainbow trout (Breinholt et al., 1995).

### **2.3.1 Phenolic Compounds**

Phenolic compounds are thought to possess both antioxidative and antiinflammatory properties, and have been reported to inhibit mutagenicity in vitro as well as in vivo. Some of these compounds inhibit carcinogenicity in animals and humans (Stavric, 1994), and some have also been shown to modulate various key cellular enzyme functions (Ho, 1992). One phenolic compound that has been studied in recent years is ellagic acid (EA). EA is a phenolic bislactone and a natural product found in vegetables and fruits, such as strawberries, blank currants, grapes, raspberries and walnuts (Josephy et al., 1990; Lord et al., 1989; Rossi et al., 1991). EA inhibits in vitro and in vivo genotoxicity of a variety of chemical carcinogens, including polycyclic aromatic hydrocarbons, N-nitroso compounds, aromatic amines and mycotoxins (Ferguson, 1994; Josephy et al., 1990; Wilson et al., 1992)

In studies conducted by Cardador et al. (2002), they identified that the greatest inhibitory effect of phenolic compound present in beans occurred when the phenolic extract (PE) was incubated with AFB1, independent of the first or second incubation in a two-stage incubation protocol. This suggests that PE could be interacting directly and non-enzymatically with the proximate and/or ultimate mutagen (AFB1 8,9 – epoxide), or forming a complex between the phenolic compounds and AFB1, thereby reducing the bioavailability of AFB1. Besides, the lowest effect could be mediated by inhibition of the mutagenic metabolism to the ultimate mutagen (AFB1 8, 9 – epoxide) or scavenging the electrophonic metabolites.

### **2.3.2 Flavonoids**

The flavonoids are glycosides with a benzopyrone nucleus. The flavones have a double bond between carbons 2 and 3. The flavonols have an additional hydroxyl group at carbon 3, and the flavanones are saturated at carbons 2 and 3. In the course of searching for antimutagenic principles from Chinese medicine, the methanol extract obtained from the whole plants of *Orostachys japonicus* showed antimutagenic activity, the active principles are flavonoids (Park et al., 1991).

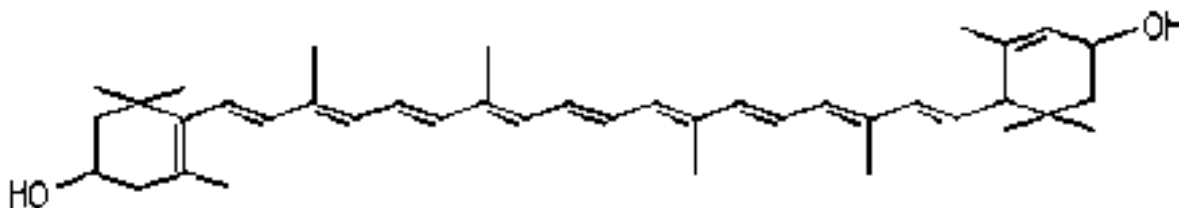
Jae et al. (1994) reported several structural features essential for the antimutagenic activity of flavonoids in the strain TA100 against AFB1 and MNNG. Those with a free hydroxyl group at positions 5 and 7 were active unless the 3 position of the C ring was glycosylated. Saturation of the 2, 3-double bond or elimination of the 4-keto group does not effect the activity for AFB1. Since the precise mechanism of the antimutagenic activity of flavonoids in the bacterial strain system is uncertain, the possible antimutagenic mechanism of flavonoids was demonstrated as inhibitory action on DNA-adduct formation through interaction with microsomal activity enzymes (Francis et al., 1989)

### **2.3.3 Chlorophyllin**

Chlorophyllin and their water-soluble salts are constituents of the human diet and have been found to be effective anticarcinogens in several animal models (Dashwood et al., 1998). Chlorophyllin is a potent antimutagen in a range of short-term genotoxicity assays in vitro and in vivo (Dashwood et al., 1998, Negishi et al., 1997). Mechanistic

studies suggest that chlorophyllin can act as an “interceptor molecule” through the formation of tight molecular complexes with carcinogens such as AFB1 (Breinholt et al., 1995). Thus, chlorophyllin may diminish the bioavailability of dietary carcinogens by impeding their absorption and by shuttling them through the fecal stream, leading to reduced DNA adduct tumor burden (Kensler et al., 1998, Breinholt et al., 1999)

#### 2.3.4.1 Natural Sources of Lutein



**Figure 2.4** Structure of Lutein



reported as lutein + zeaxanthin, making examination of specific effects of dietary lutein difficult. In terms of food sources, human metabolism, and tissue storage, lutein and zeaxanthin are similar.

**Table 2.2** Food Sources of Lutein

Food	Lutein Content (µg/100g wet wt)
Broccoli	2358
Kale	6390
Carrot	280
Spinach	3920
Tomato granulate	226
Tomato powder	39
Tomato flakes	99

*Adapted from (Huck et al., 2000)*

#### **2.3.4.2 Lutein in Health and Disease**

The first report that the yellow spot in the macula of human retinas might be a carotenoid appeared in 1945. George Wald dissected the foveal region of 10 human retinas, extracted them with chloroform, and reported that the spectrum of the yellow pigment agreed quite well with the visual estimate of the macular pigment, derived from the differences in the log sensitivity of peripheral and foveal cones (Wald, 1945). Fifty years after this observation, carotenoids were also identified in the lens of the human eye

(Yeum et al., 1995) and several years later, carotenoids were identified in virtually all of the tissues of the eye (Bernstein et al., 2002).

Dietary carotenoids are thought to provide health benefits in decreasing the risk of disease, particularly eye disease. It has been suggested that eating leafy vegetables, which are rich in lutein and zeaxanthin, may decrease the risk for eye disease called Age-Related Macular Degeneration (AMD) (Seddon et al., 1994). AMD is the most common cause of irreversible vision loss and legal blindness among older people. It is a degenerative condition of the region of the retina that is responsible for central vision. These carotenoids are concentrated in the inner retinal layer of the macula, where the concentration is high and variable (Bernstein, 2002). Evidence from human studies suggest that dietary intake of carotenoids can lead to their accumulation in the retina and, therefore, may provide protection against retinal degeneration.

An inverse relationship between lutein intake and colon cancer was found for all subjects, meaning that the more lutein-containing foods that were consumed, the less of a risk of colon cancer. The study, led by Martha Slattery of the Health Research Center in Salt Lake City, Utah, looked at 1,993 subjects ages 30 to 79 years who had been diagnosed with colon cancer, and a control group of 2,410 people who did not have cancer (Slattery et al., 1988). Participants were asked to report the foods they had eaten during a specific time period two years before or two years prior to their diagnosis. Nutrients contained in the foods were then calculated using a database. Of all the carotenoids investigated, only lutein and zeaxanthin showed a protective effect against

colon cancer. The antioxidant effect of lutein and zeaxanthin is linked to their biochemical effectiveness as scavengers of oxygen radicals, as well as their reaction with cell membranes in the colon, which are susceptible to carcinogenesis.

#### **2.3.4.3 Antimutagenicity of Lutein**

Xanthophylls are excellent antioxidants with antimutagenic and anticarcinogenic properties. In a study by Gonzalez et al. (1997), they used the Salmonella plate incorporation test to examine the effect of xanthophylls extracted from Aztec Marigold on AFB1 mutagenicity, using tester strain YG1024. As a result, they found pure lutein and xanthophylls from Aztec Marigold flower inhibited the mutagenicity of AFB1 in a dose-dependant manner. In another similar study held by Gonzalez et al. (1997), they used the Salmonella typhimurium tester strain YG1024 in the plate-incorporation test to examine the antimutagenicity of xanthophylls extracted from Aztec Marigold (*Tagetes erecta*) on 1-nitropyrene (1-NP) mutagenicity. Lutein and xanthophylls from Aztec Marigold (pigments for poultry and human use) inhibited mutagenicity of 1-NP in a dose-dependent manner. Furthermore, lutein has the characteristic of antimutagenicity against benzo[a]pyrene and 2-amino-3-methylimidazo[4,5-f]quinoline (Rauscher et al., 1998).

#### **2.4 Evaluation of Mutagenicity**

The Salmonella mutagenicity test (Ames test), developed by Bruce Ames at the university of California, Berkeley, has been widely used for detecting the mutagenicity of variety of chemicals. The types of mutation detected are the base-pair substitution and the

frame-shift. Both of these alterations cause changes in the reading of the DNA and result in three kinds of coding error: 1) Sence-this is where the codon has been changed but still reads for the same protein, 2) Non-Sence-the codon had been changed and makes no sense and reading stops, and 3) Missence-the codon reads for a different protein. The principle of the Ames test is to culture one or more histidine-dependent *Salmonella typhimurium* strains that are exposed to the potential mutagen in the his<sup>-</sup> culture medium. The potential mutagen reverts the bacteria back to histidine-independent by inducing a mutation in the histidine gene. The results can be obtained in 48 hours. The greater the number of induced revertants per plate relative to the control, the greater the probability that the revertants were induced by the test mutagen.

#### **2.4.1 Tester Strains**

A set of histidine-dependent strains is used for mutagenicity testing. Each tester strain contains a different mutation that can code for histidine biosynthesis in the open. Tester strains TA97, TA98, TA100 and TA102, which are commonly used in aflatoxin-induced mutagenic studies (Draughon and Childs, 1982; Schroeder et al., 1985; Lawlor et al., 1985; Jorgensen et al., 1990), contain a mutation (rfa mutation) that makes the lipopolysaccharide barrier coating the surface of the bacteria more permeable to large molecules such as benzo [a] pyrene that do not penetrate the normal cell wall (Ames et al, 1973). Another mutation (UVRB) in these tester strains (except TA102) is a deletion of a gene coding for the DNA excision repair system resulting in greatly increased sensitivity in detecting many mutagens (Ames, 1973). In order to increase their sensitivity,

these tester strains contain an R-factor plasmid PKM101, which enhances an error-prone DNA repair system and increases the chemical and spontaneous rate of the test strains (Ames and McLann., 1981; Levin et al., 1982).

Now the recommended strains for use in the Ames assay are tester strains TA98 and TA100 originally derived from *S. typhimurium* LT2 (Gatehouse et al., 1994). In 1998 the histidine mutation which consists of a shifted pairing that occurs in repetitive sequence or “hot spots” (in this case, repetitive GC) was found in the sequence coding for histidinol dehydrogenase. Furthermore, Isono and Yourho (1974) reported that tester strain TA98 detects various mutagens which cause a frameshift type of mutation that restore the right sequence for histidine biosynthesis. The histidine mutation in TA100 is found in the sequence coding for the first enzyme in the histidine biosynthesis pathway. This mutation substitutes histidine independent GCGCGC for histidine dependent GCATGC (Barhes et al., 1982). As a result, TA100 tester strain detects mutagens that cause a base-pair substitution type of mutation, which restores the right sequence for production of histidine. When bacteria have been received, when a new frozen or lyophilized permanent has been opened, right before performing a mutagenicity test, when spontaneous revertants fall out of normal range, or when the sensitivity to standard mutagens has become weakened or lost, genotypes of tester strains should be confirmed. For these purposes, a series of biochemical tests is applied. The tests include: (a) confirmation of Histidine requirement, (b) testing of UV light sensitivity (UVRB mutation), (c) testing of crystal violet sensitivity (rfa mutation), (d) testing of antibiotic,

and (e) resistance factor (R-factor).

#### **2.4.2 Spontaneous Reversion**

Spontaneous reversion of the tester strains to histidine independence is measured in mutagenicity assays and expressed as the number of spontaneous revertants per plate. There is variability in the number of spontaneous revertants from one experiment to another and from one plate to another, but they should be relatively consistent within a lab. The following ranges are based in historical values and are therefore more reliable. The revertants/plate (-S9) for tester strains are TA97 (90-180), TA98 (30-50), TA100 (120-200), and TA102 (240-320) (Maron and Ames, 1983). The numbers may vary slightly on plates containing S9 mix.

Sometimes different ranges of spontaneous reversion may be observed but there should not be extreme fluctuation from one experiment to the next. If a deviation is obviously outside the acceptable range, it is an indication that the genetic characteristics of the strain in question (or the growth medium) should be tested; while if the spontaneous reversion is high, it may indicate contamination or the accumulation of back mutation by repeated sub-culturing (Maron and Ames, 1983). A decrease in the number of spontaneous revertants accompanied by sensitivity to ampicillin and insensitivity to the appropriate diagnostic mutagens are indications of partial or complete loss of the R-factor. Spontaneous reversion is independent from the initial number of bacterial cells plated but it is influenced by the histidine concentration.

### **2.4.3 Metabolic Activation**

Many pro-mutagens and pro-carcinogens remain inactive until enzymes are transformed into electrophilic species capable of binding to DNA leading to mutation (Njapau, 1999). In the Ames test, a phenobarbital 5,6 –benzoflavone or Aroclor 1254-induced rat liver (S9) is routinely used to effectively enhance the bioactivation of different classes of carcinogens (Ames et al., 1973; Kier et al., 1974). The S9 suspension contains several microsomal enzymes, including the mixed function oxidase system (cytochrome P450), which are responsible for the transformation of the parent compound into reactive metabolite. Aflatoxins require metabolic activation by specific microsomal and nuclear cytochrome P450 associated enzymes to produce the highly reactive AFB1-8, 9-epoxide, the ultimate carcinogenic compound (Mehta et al., 1993). The AFB1-8, 9-epoxide readily binds to nucleophilic centers in many cellular macromolecules such as proteins and the N-7 guanine of DNA (Groopman et al., 1992).

### **2.4.4 Types of the Mutagenicity Test**

Plate incorporation and spot test are the most commonly used methods in the Ames test. According to the principle of the Ames test, the plate incorporation test consists of combining the test compound, the bacterial tester strain, and S9 mix in soft agar, which is poured onto a minimal agar plate. The mixture is incubated at 37 °C for 48 h, and then revertant colonies are counted. The spot test is a variation of the plate incorporation test. In this test, the mutagen is left out of the agar overlay, and is applied to the surface of the minimal agar plate after it has been seeded with the bacterial tester strain and S9 mix.

Compounds tested for mutagenicity are routinely dissolved in dimethyl sulfoxide (DMSO) when they are not soluble in water. DMSO is used because of its capability of dissolving a wide range of chemicals. In addition, it is non-toxic to the bacteria, it does not affect the microsomal enzymes, and it is miscible in the molten top agar (Szmant, 1971).



## **CHAPTER 3. EVALUATION OF LUTEIN AND PROTEIN IN OZONE TREATED CORN**

### **3.1 Introduction**

Corn is currently the third most planted field crop after wheat and rice. The bulk of corn production occurs in the United States, Peoples Republic of China, and Brazil, which together account for 73% of the annual global production of 589.4 million tons (FAO, 1998). In Louisiana, corn ranks fourth after sugarcane, cotton, and rice as an agricultural commodity. Corn is a very important commodity not only to the States, but also to the whole world. However, in most warm and humid regions the corn crop is highly susceptible to fungal invasion and aflatoxin production. Current estimates show that in 1998, 25% of corn fields in Louisiana were rejected or never harvested due to suspected aflatoxins contamination. Moreover, the presence of aflatoxins in food and feeds poses serious problems in human and animal health. Aflatoxin B1 is the most potent of four naturally occurring aflatoxins. Because of health and economic problems, the poison has been the focus of considerable research since its discovery (McKenzie, 1997).

In order to limit human exposure to aflatoxins, prevention and control programs are constantly being studied and evaluated to get more efficient and safer methods. There are several kinds of methods in decontamination such as physical, chemical or biological methods. The chemical methods are currently the most practical approaches to inactive aflatoxins. Ozone treatment is one method that has been studied. Ozone, a powerful

oxidizing agent, reacts across the 8,9-double bond of the furan ring (Samarajeewa et al., 1990). Ozone was able to reduce aflatoxin in cottonseed meal and peanut meal (Dollear et al., 1968; Dwarakanath et al., 1968). They reported that 91% of the total aflatoxins were destroyed in 22% moisture corn after 2 hours, while the reduction in the peanut meal was only 78% after exposure to ozone for 1 hour. In 1997, McKenzie reported that aflatoxins in corn could be reduced by 95% after being treated with 200mg/min ozone for 92 hours. Prudente and King (2002) observed a 92% degradation of aflatoxin by ozonation.

Corn is a rich source of flavonoids, polyphenols and carotenoids (Rooney and Serna-Saldivar, 1987). The occurrence of these antioxidants does not only decrease pest infestation (Gueldner et al., 1992), but also directly reduces aflatoxin levels in the grains (Norton, 1997). Flavonoids, carotenoid and polyphenols mitigate the toxic and or mutagenic effects of aflatoxin (Park et al., 2004; Gonzalez de Mejia et al., 1997; Cardador-Martinez et al., 2002). Pure  $\alpha$ -carotene and lutein, both of which occur in corn, reduced the mutagenic effect of aflatoxin to 2% that of control (.Gonzalez de Mejia et al., 1997).

Although ozonation has been proven to be an effective method for decontamination of aflatoxin in corn, its suitability and acceptability have yet to be evaluated. This study therefore set out to isolate and identify lutein, to verify lutein extracts from corn antimutagenic effects against aflatoxin, and to determine the effects of ozonation on its levels in clean and contaminated corns. This study also aimed to determine the effect of

the ozone process on the protein composition of corn.

## **3.2 Materials and Methods**

### **3.2.1 Chemicals**

Ethanol, potassium hydroxide, hexane, acetone (HPLC grade), petroleum ether, and methanol (HPLC grade) were obtained from Fisher (Fairlawn, NJ). Ampicillin, D-biotin, magnesium sulphate, sodium ammonium phosphate, citric acid monohydrate, L-histidine, tetracycline, magnesium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate,  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), glucose-6-phosphate, glucose, Sodium chloride, potassium chloride, lutein standard, pure aflatoxin standard and butylated hydroxy toluene (BHT) were purchased from Sigma Chemical Co. St. Louis. Electrophoretic gels (4-12% Bis-Tris gels, catalog no. NP 0321), lithium dodecyl sulfate sample buffer (catalog no. NP 0007), molecular weight marker (catalog no. LC 5677), acetic acid, running buffer (catalog no. NP 0002), and staining solutions (catalog no. 46-016) were obtained from Invitrogen (Carlsbad, CA), MO. Bacto agar was obtained from Difco Laboratories (Detroit, MI). Oxoid nutrient broth NO.2 was sourced from Unipath LTD (Basingstoke, Hampshire, England). Rat liver post-mitochondrial supernatant (S9 mix) was purchased from Molecular Toxicology Inc., (Boone, NC). Bacterial tester strain TA100 was kindly provided by Dr. Bruce Ames (UC Davis, CA)

### **3.2.2 Corn Sample**

Corn Samples were kindly provided by Dr. Kenneth S. McKenzie of Lynntech, Inc., College Station, Texas. The samples were treated at Lynntech, Inc. as follows. Ten kilograms each of corn sample with and without aflatoxin contamination was treated with ozone. Corn sample was placed into a 30-gallon polyethylene reactor with false bottom. A 10-15" headspace was allowed to achieve even ozone dispersion through the corn. The reactor lid was fitted with 1/4" Teflon bulkheads. Ozone gas, 10-12 wt%, was flowed in through the top at approximately 2 L/min. A 2.5 L/min vacuum was placed at the bottom. All corn samples were treated for 96 hours at 12-15 hour intervals with mixing occurring every 30 hours. The treatment protocol included untreated clean corn, ozone-treated clean corn, naturally contaminated corn and ozone-treated naturally contaminated corn.

### **3.2.3 Sample Preparation**

Ten (10) kilograms of corn sample from each treatment was ground using a Romer Hammer Mill to produce three subsamples that were further ground further using a Brinkmann mill to pass a No. 20 mesh sieve. Samples were transferred to clean plastic bags, labeled and were stored at 4°C until further analysis.

### **3.2.4 Extraction of Lutein**

Lutein extraction was a modification of the procedure of Moros et al. (2002). Triplicate ground corn samples, 20g of each treatment type, were each placed in 500 ml Erlenmeyer flasks and 120 ml of 0.1% (w/v) BHT-EtOH solution was added to each flask.

The flasks were sealed with screw caps and placed in a 75°C water bath for 5 min. The flasks were then removed from the water bath and 4 ml of 80% KOH was added to each flask. Samples were then shaken for 2 min and returned to the water bath for 10 minutes for saponification to occur. After the samples were saponified, the flasks were immediately placed into an ice bath to cool, and then 60 ml of cold deionized water was placed into each flask, followed by 30 ml of hexane, followed by shaking. Then the sample solutions were centrifuged at 2500 rpm for 10 min. The top hexane layer was removed with a Pasteur pipet and added to a separate 250 ml Erlenmeyer flask. The hexane extraction was repeated until the top layer was colorless. All hexane extracts were combined in the same flask. The hexane was evaporated in a stream of nitrogen passed into the flask until dry. The residue was then solubilized in 5 ml of mobile phase (methanol/acetone 90:10) and stored at -20°C for HPLC analysis and the Ames test.

### **3.2.5 HPLC**

The analytical HPLC system consisted of a reversed phase Supelco (Bellefonte, PA) Discovery C18 column (id 3 mm × 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium chromatography manager. A guard column (4mm×23mm) containing the same packing materials as the C-18 column was installed ahead of the C18 column. The mobile phase was a mixture of methanol and acetone at a ratio of 90:10. The flow rate was 1.0 ml/min during the entire run. The inject volume of all samples was 20 ul. The detector was set at 456 nm. The analyses were performed in triplicate. The contents of lutein in the corn were calculated by comparing the peak area

with that of standard lutein using a standard curve.

### **3.2.6 Protein Analysis**

#### **3.2.6.1 Extraction of Protein from Corn**

Corn flour (200 g) was defatted by extraction with 500 ml of petroleum ether at 21°C overnight in a 1000 ml Erlenmeyer flask. The defatted flour was air-dried under a hood, extracted with stirring with 1000 mL of 70% ethanol containing 0.5 M NaCl in water for 4h at 21 °C, and refrigerated until equilibrated to 4°C. Then the mixture was centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was decanted into a container. The ethanol was removed under vacuum by rotary evaporation, and the protein solution was lyophilized. Protein concentration in the powder was determined by nitrogen analysis ( $N \times 6.25$ ) (2410 Nitrogen Analyzer, Perkin-Elmer, Shelton, CT). All assays for each treatment sample were done in triplicate.

#### **3.2.6.2 Electrophoresis of Corn Protein Mixture**

SDS-PAGE electrophoresis was carried out following Invitrogen protocol (Carlsbad, CA). Lyophilized corn protein extract powder at 1 mg/mL was dissolved in sample buffer. Ten microliters of the protein sample was added to 25 uL of sample buffer and 65 uL of deionized distilled water following instructions from the gel's manufacturer. Electrophoretic separation was carried out using a Mini-VE electrophoresis unit (Amersham Pharmacia Biotech, Piscataway, NJ). The gel was stained using Novex Colloidal Blue. Samples were run in duplicate.

### **3.2.7 Evaluation of Antimutagenicity of Lutein**

The antimutagenicity of lutein extracts were tested using the standard plate incorporation Salmonella/microsomal mutagenicity assay as described by Maron and Ames (1983). Working in a laminar flow hood disinfected with 80% alcohol, a single colony was picked from an ampicillin master plate and placed in 40 ml of sterile nutrient broth in an Erlenmeyer flask. The flask was lightly capped to allow airflow and placed in a gyratory water bath, set at 200-250 rpm and 37°C, for 12-14 hours. In this test, TA100 test strain was used. After incubation, growth was confirmed by checking the turbidity using a spectrophotometer at 650 nm. Sterile Oxoid Broth No.2 was used as a blank. Absorbance readings in the range of 0.75-0.85A indicated an optimal cell density of  $1-2 \times 10^9$  bacterial cells/ml.

S9 mix was prepared just before commencement of the test. All apparatus and solutions used were sterilized and all operations were conducted under a laminar flow hood. Before preparing the S9 mix, lutein extracts, which were dried with a stream of nitrogen, were reconstituted in dimethyl sulfoxide (DMSO) and diluted (5, 25 and 625 times). Lutein standard was also solubilized in DMSO (0, 0.002, 0.02, 0.08, 2, and 10 ug/plate). The concentrations of aflatoxin B1 in DMSO used in each plate for the AFB1 standard were 10, 50, 100, 250, and 500 (ng/plate). During the assay the S9 mix was kept on ice. AFB1 (500 ng) was combined with 0.2 ml histidine/biotin solution, 0.1 ml TA100, 0.1 ml lutein standard/extracts and 0.5 ml S9 mix with 2 ml soft top agar. The mixtures

were vortexed and poured onto a minimal glucose agar plate and incubated at 37°C for 48 hours. The number of revertants was counted and was compared against natural revertants and AFB1 standard. All assays were done in triplicate.

### **3.2.8 Statistical Analysis**

Each of the control and treatment groups were replicated three times at the same time. Student's t- test procedure (Excel Data Analysis, Microsoft Inc.,Seattle, WA) was used to compare the levels of lutein in the treated and untreated corn. In the Ames test, the statistical significance of the differences between the lutein standard and lutein extract was also determined using the student's t-test. Significant difference among means was considered at  $P \leq 0.05$ .

## **3.3 Results and Discussion**

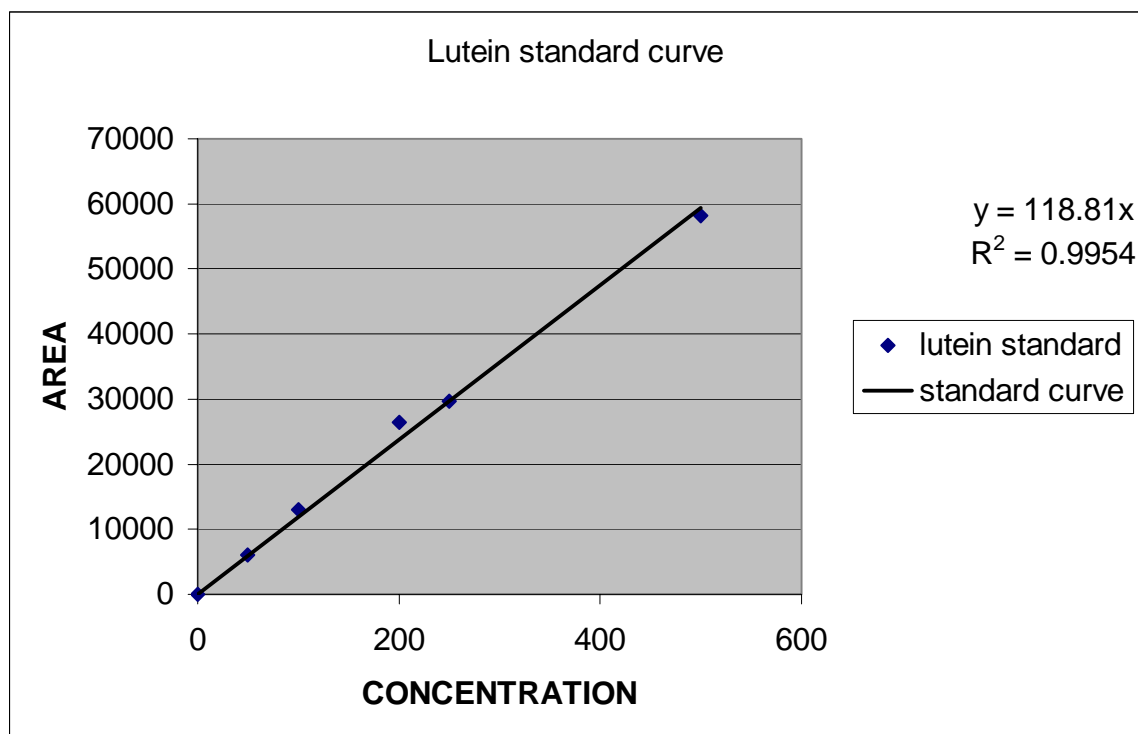
### **3.3.1 Lutein Determination**

The standard curve of lutein shown in Figure 3.1 was constructed by plotting HPLC peak absorbance area vs. concentration of the xanthophylls in the injected standard. The elution profile of the lutein standard with the C18 column and reverse-phase chromatography is shown in Figure 3.2.

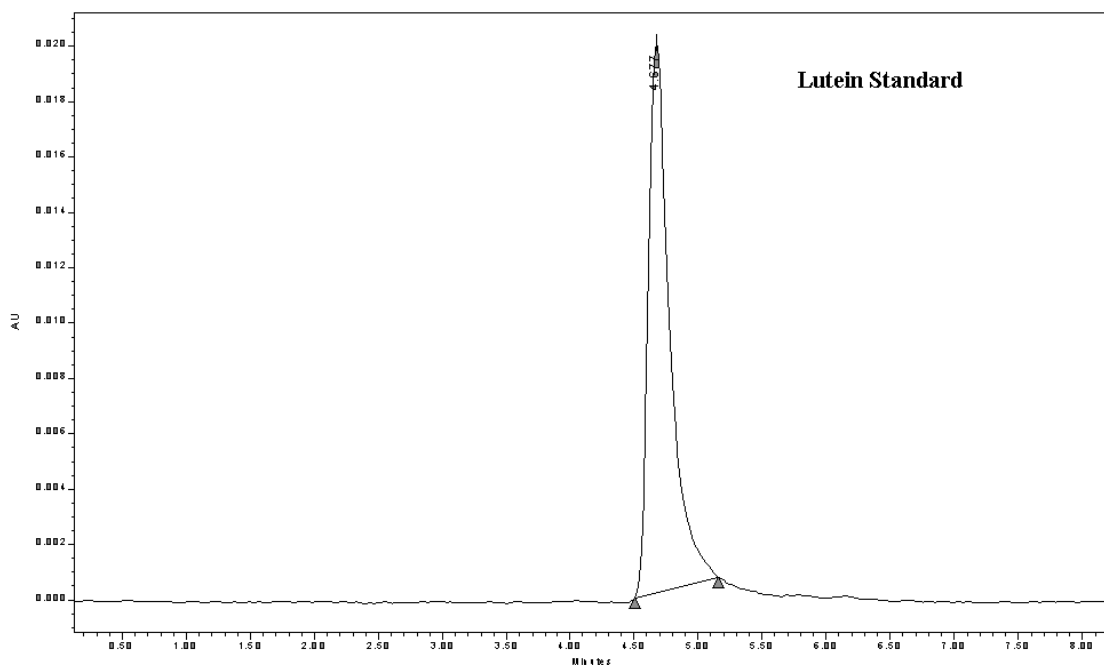
The retention time was about 4.6 min for lutein. With this HPLC column, the more polar, the less the retention time. In contrast, the order of retention time is reversed with the normal silica columns. Lakshminarayana et al. (2005) and Li et al. (2002) reported that the retention time of lutein standard using a C18 column and a similar mobile phase was about 4.5 min.



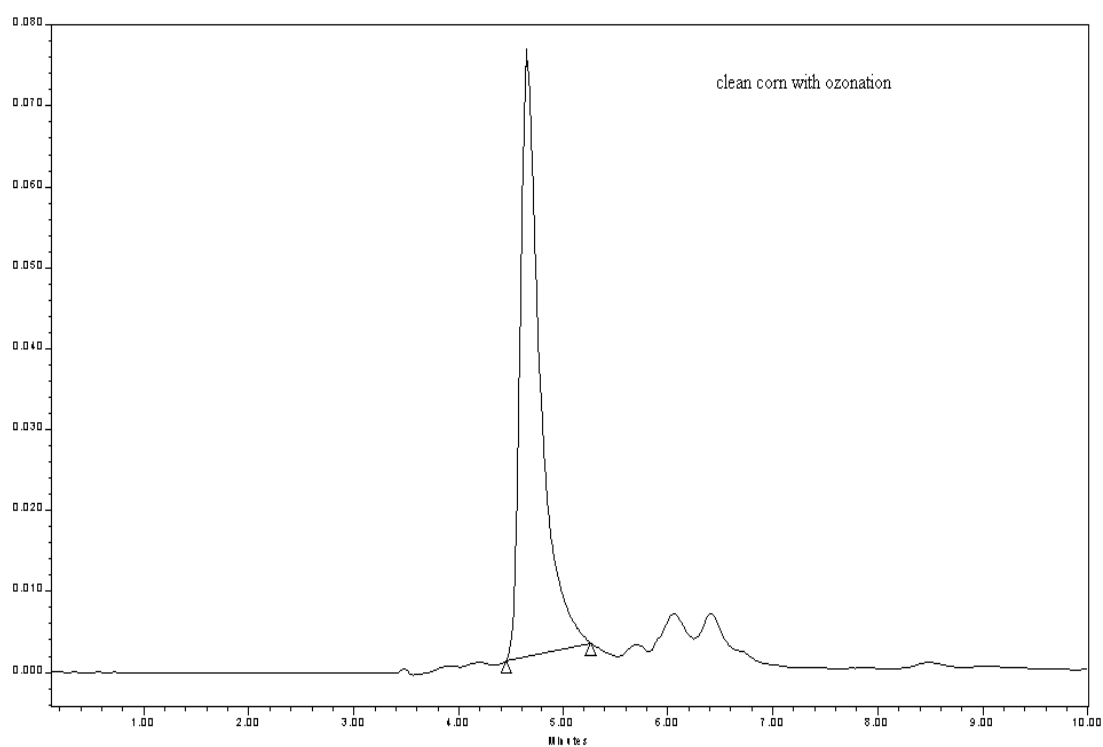
Figures 3.3, 3.4, 3.5, and 3.6 show chromatograms of the different corn samples after one BHT-EtOH extraction and three hexane washes as described in the methods. The peaks were well separated by the C18 column. Identification was based on the retention time and spectra of absorbance maxima of a particular peak compared to lutein standard.



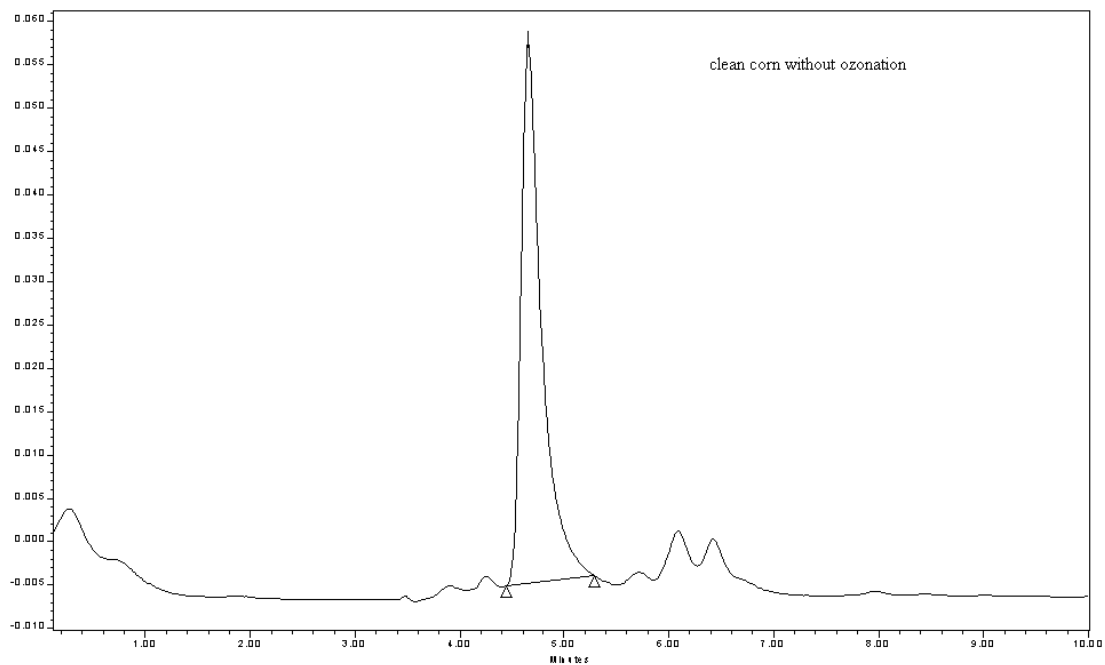
**Figure 3.1** Standard curve of lutein detected at 456nm



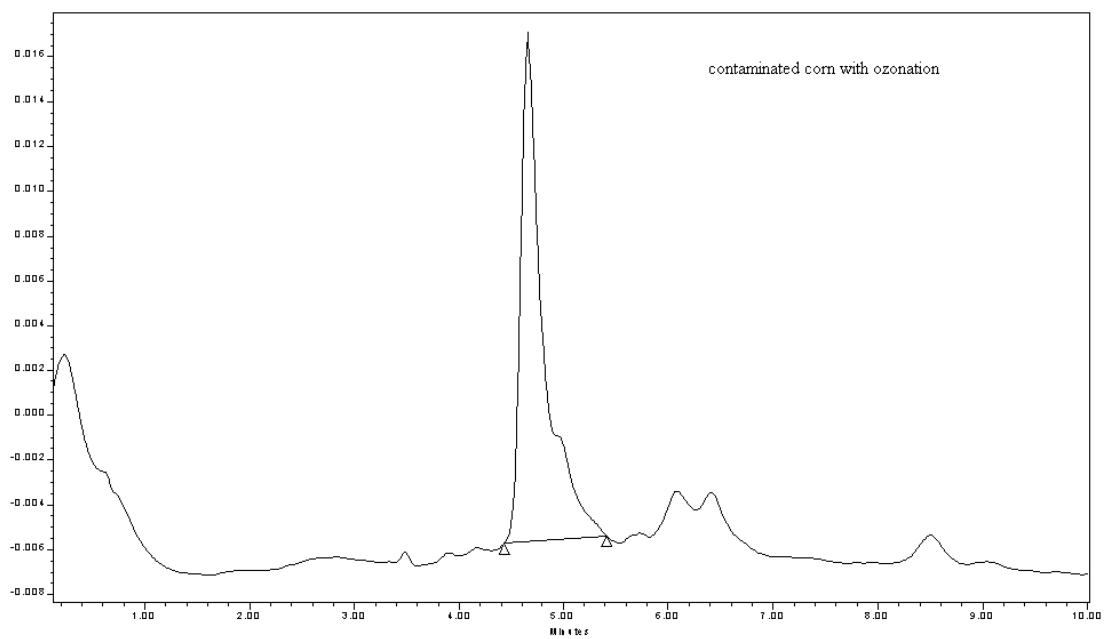
**Figure 3.2** Chromatogram of lutein standard



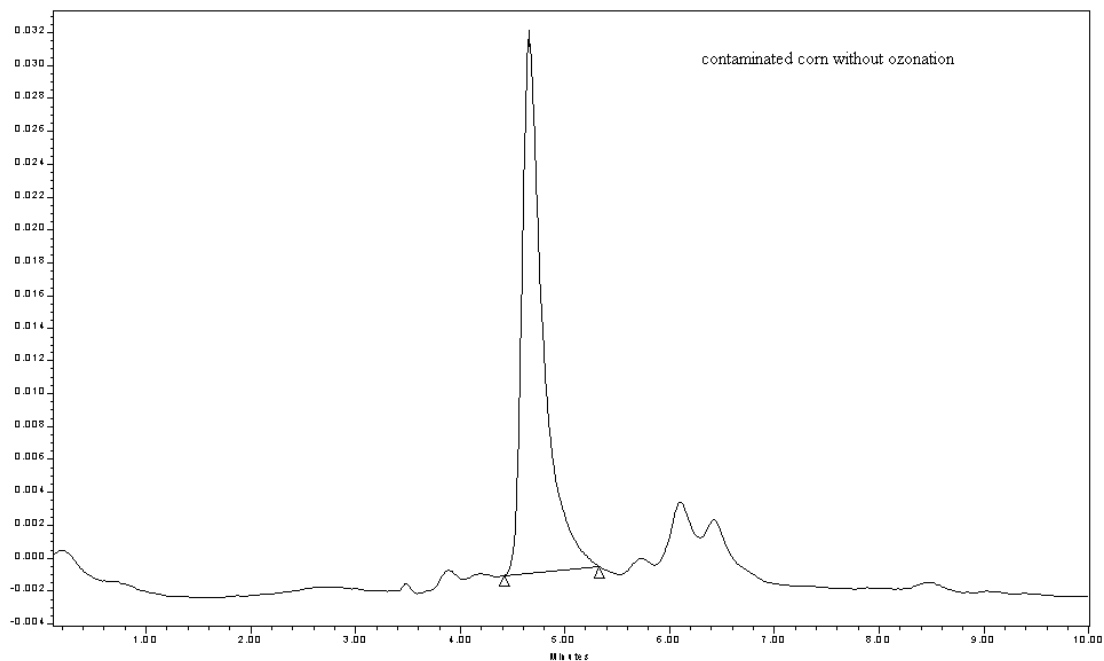
**Figure 3.3** Chromatogram of lutein extract in the clean corn with ozonation



**Figure 3.4** Chromatogram of lutein extract in the clean corn without ozonation



**Figure 3.5** Chromatogram of lutein extract in the contaminated corn with ozonation



**Figure 3.6** Chromatogram of lutein extract in the contaminated corn without ozonation

Lutein samples were identified at a retention time of 4.6 min with absorbance maxima at 456 nm. According to Moros et al (2002) and Lakshminarayana et al. (2005), the next two peaks may be zeaxanthin and chlorophyll, respectively. However, analyses of zeaxanthin and chlorophyll standards were not done.

All these peaks of different corn samples looked almost similar to each other. But the concentrations were totally different. Table 3.1 shows the content of lutein in the different corn samples.

After treatment, the total amount of lutein changed. The amount of lutein in the clean corn changed from 22.75 ug/g to 28.36 ug/g, while the amount of lutein in the contaminated corn changed from 16.42 ug/g to 11.69 ug/g. The content of lutein in the treated clean corn was higher than that of lutein in untreated clean corn. However, the result was reversed in the contaminated corn.

**Table 3.1** Lutein contents (ug/g) of different corns

Sample	Lutein content (ug/g corn)
A	28.36±0.35
B	22.75±0.11
C	11.69±0.12
D	16.42±0.19

A: clean corn with ozonation, B: clean corn without ozonation

C: contaminated corn with ozonation, D: contaminated corn without ozonation

Statistical analysis showed that the level of lutein in treated clean corn was significantly higher than that in untreated clean corn with  $P \leq 0.001$ . Likewise, the level of lutein in the treated contaminated corn was significantly lower than that in untreated contaminated corn with  $P \leq 0.001$ .

The average amount of lutein in the corn in Moros's report (2002) was 14.68 ug/g, which was a little bit lower than the result of clean corn without ozonation. When Moros's extraction step was repeated five times, the amount of xanthophylls was 22.81 ug/g. The reason for the greater amount of xanthophylls in the treated corn may be that some xanthophylls or lutein are bound to other compounds such as fatty acids, protein and starch or trapped in the corn solid. Because of ozonation, lutein may have been released from those compounds.

Lutein ester is one kind of these bound products (Antony et al., 2001). KOH was used to enrich free lutein from lutein ester by saponification. In the research of Moros

(2002), the content of lutein in whole corn was compared with corn gluten meal. As a result, total xanthophylls concentration was  $145.91 \pm 2.06$  ug/g corn gluten meal, about 7.2 times higher than whole corn assayed under similar conditions. Moreover, the protein content of gluten meal is about 60% (dry basis) compared to 7.6% protein in whole corn, about 7.9 times higher. This suggests that the xanthophylls are probably bound to a protein, probably zein. It was pointed out that if hexane was used to get the deoiled corn, about eighty five percent of the xanthophylls remained in the corn (Moros et al., 2002). Fifteen percent of xanthophylls may be lutein ester in the oil, while the rest of the lutein may interact with zein by hydrophobic bonds. Zein which is ethanol soluble is classified as  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -zein on the basis of differences in solubility and sequence (Esen, 1986). The structure of zein in the corn is the key point of combination with lutein. There are 56.7%  $\alpha$ -helix, 7.1%  $\beta$ -sheets, 8.2% coil and 28% not determined in the  $\alpha$ -zein (Cabra et al., 2005). The model reported by Argos et al. (1982) indicated that the repetitive sequence of the zein-forming  $\alpha$ -helix is highly hydrophobic, i.e., rich in leucine, and also includes phenylalanine and tyrosine. Thus, it is reasonable to speculate that such a hydrophobic  $\alpha$ -helix region in the zein has a high affinity for lutein molecules. When corn was treated with ozone, the ozone possibly destroyed zein so as to release lutein. However, in the contaminated corn the amount of lutein in the treated corn was less than that in the untreated corn. That is to say, because of contamination, zein and lutein structure was changed. Lutein was more accessible to ozone in the contaminated corn. Zein can be used by *Aspergillus flavus* as a nitrogen and carbon source (Mellon et al.,

1998). They reported that presence of zein as a supplement in chemically defined culture media stimulates aflatoxin production up to 10-fold and its presence as a sole nitrogen source, together with a rapidly accessible carbon source, stimulates aflatoxin production by 8-fold over reference cultures grown on a chemically defined medium.

### **3.3.2 Protein Analysis**

Table 3.2 shows the content of protein in different corn samples. The content of protein in the treated corn was lower than untreated corn. In the clean corn, the percent of protein of treated corn was 10.56%, while in the untreated the percent was 12.16%. In the contaminated corn, it was similar to the clean corn. Corn contains 70-75% starch, 5% lipids (triglycerides), and 11% protein by weight (Bewley et al., 1978). The protein content of our sample was similar to that of the literature. Zein comprises 50% of the total protein component in the mature seed. Those results suggest that ozone can destroy the protein. Meanwhile, it is a further explanation that lutein may bind to protein in corn. Figure 3.7 shows the result of SDS-PAGE.

In the study by Cabra (2005), SDS-PAGE results usually divide  $\alpha$ -zein into two groups based on their migration (Z19 and Z22). However, the apparent molecular mass of the peptides was often different in the various reports because of the use of different gel systems, standard proteins, and corn varieties. Apparent molecular masses of 18-24k for Z19 and 21-26k for Z22 have been reported by different authors (Wilson, 1991). In fact,  $\alpha$ -zein is a mixture of a large number of proteins. Wilson (1991) showed at least 15

components in  $\alpha$ -zein by RP-HPLC serial analysis. Our SDS-PAGE shows two bands at approximately 22k Da and 26k Da (Figure 11). From the gel, there were no changes in protein type. Some bands were lighter, when the corn was treated with ozone. The density of lighter band was lower, and maybe the concentration of that protein was lower also. The contaminated corn protein may have been more susceptible to ozone degradation. Bands in 4 and 9 were the lightest, and the concentration of protein, which was 8.85% in the treated contaminated corn, was lowest. Bands in 2 and 7 were lighter, so the protein content of 10.56% in the treated clean corn was between the amount of untreated and treated contaminated corns. Results of experiments in which amino acids were exposed to ozone showed that the most susceptible amino acids were cysteine, tryptophan, methionine, and histidine (Pryor et al., 1984). There are specific instances of reaction of each of these amino acids in proteins including cysteine in glyceraldehydes-3-phosphate dehydrogenase (Knight, 1984), tryptophan in lysozyme (Kuroda, 1975), methionine in glycophorin (Banerjee and Mudd, 1992), and histidine in glutamine synthetase (Berlett et al., 1996). It has been suggested that the reaction rate constant of ozone with amino acids in solution is the same when the amino acid is in the peptide bond (Pryor and Uppu, 1993). Kasai (1993) reported that the ozonolysis reaction caused changes in casein that extended beyond the destruction of aromatic amino acid residues. Sixty eight percent of phenylalanine was decomposed by ozonolysis of casein, but all tyrosine, tryptophan and histidine, 50% of cystine, 20 to 25% of threonine, serine, valine, isoleucine, lysine and arginine and 10 to 20% of proline, glycine and alanine were also destroyed. All of



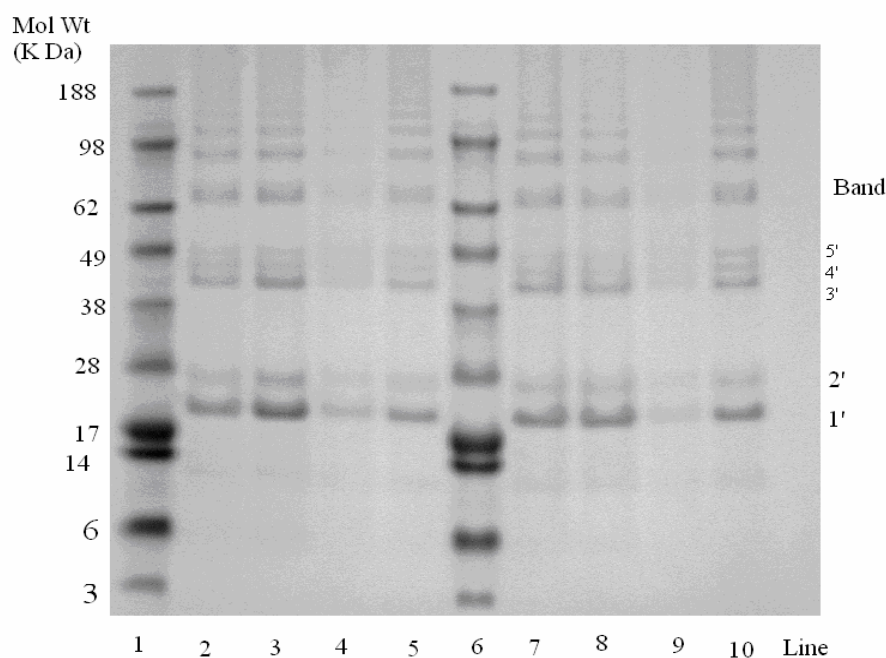
methionine was oxidized to methionine sulfone.

**Table 3.2** Protein content in the different corn samples

Sample	Protein content
A	10.56%
B	12.16%
C	8.85%
D	12.04%

A: Clean corn with ozonation, B: Clean corn without ozonation

C: Contaminated corn with ozonation, D: Contaminated corn without ozonation.

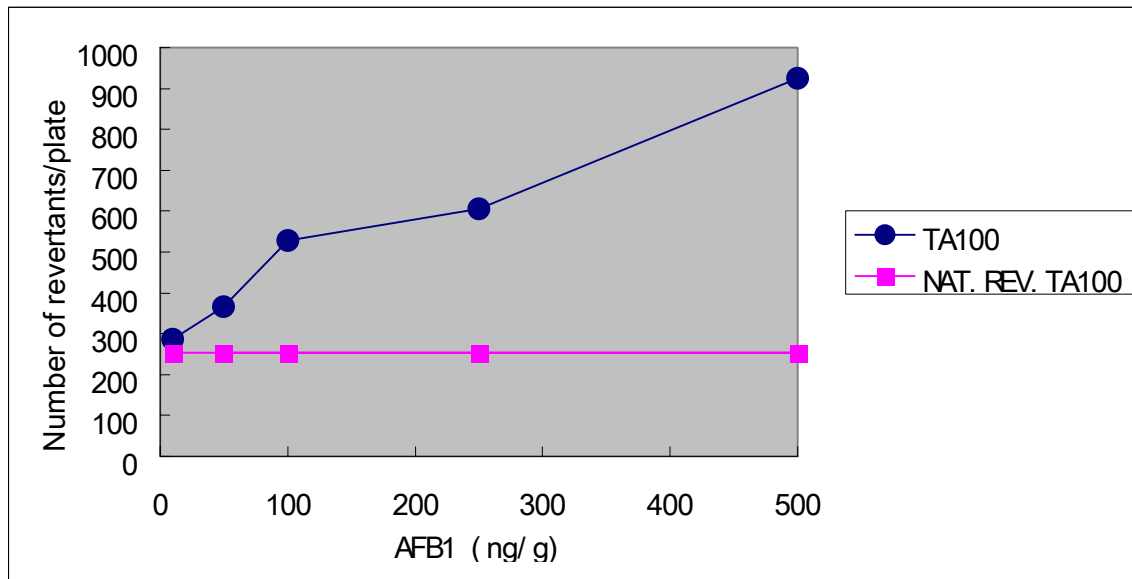


**Figure 3.7** SDS-PAGE of protein extracted from corn. Lane 1 and 6, molecular weight standards; Lane 2 and 7, clean corn with ozonation; Lane 3 and 8, clean corn without ozonation; Lane 4 and 9, contaminated corn with ozonation; Lane 5 and 10, contaminated corn without ozonation.

### 3.3.3 Evaluation of Antimutagenicity of Lutein

The antimutagenic potential of lutein extracted from corn was evaluated to determine the potential risk the ozonation process might have on the final product. Figure

3.8 shows the dose response curve for AFB1 standard. Qin and Huang (1985) reported that with a concentration of 500 ng AFB1/plate in TA 98, the mutagenic potency was 1,117 revertants/plate. On the other hand, Bhattacharya et al. (1987) found that with a concentration of 0.4 ug AFB1/plate in TA100, the mutagenic potency was  $2,386 \pm 158$  revertants/plate, 2.5 times more compared with our results. Prudente (2001) observed that with a concentration of 500 ng AFB1/plate, the mutagenic potency was about 1,100 revertants/plate, which is similar to our result 925 revertants/plate. These results indicate that different test strains have the different efficiency at detecting the mutagenicity of AFB1, so standard curves should be done every time.



**Figure 3.8** Standard curve for pure AFB1 using *Salmonella typhimurium* tester strains TA100 with metabolic activation. (Values are means of three replicates).

Lutein standard and lutein extracts were investigated for mutagenic potential (Table 3.3 and 3.4). Table 3.5 shows the concentration of lutein extracts in different dilutions. When the concentration of lutein standard were 0.2, 0.8, and 10 ug/plate, the number of revertants was similar to that of the negative control (natural revertants). The number of revertants for lutein standard at the concentrations of 0.2, 0.8, 10 ug/plate were 254, 261, and 264 respectively (Table 3.3). Although some of the lutein extracts (Table 3.4) had a slightly higher number of revertants than the natural of revertants, they were still close to  $253 \pm 23$ . It can therefore be stated that the Ames test showed that purified lutein and lutein extracts from corn do not induce mutagenicity in TA100 using the plate incorporation method. Kruger (2002) investigated two formulations of purified lutein, encapsulated beadlet containing 10% purified lutein and non-encapsulated purified lutein. For both sample and for all five tester strains, the number of revertants was not increased. These recent findings are consistent with a number of previous studies demonstrating the absence of any mutagenic effect of lutein using the Ames test in *S. triphimurium* strains (Gonzalez de Mejia et al., 1997 a, b; Rauscher et al., 1998).

The dose of 500 ng AFB1/plate was chosen for the antimutagenicity studies. The antimutagenic effect of lutein standard and lutein extracts on AFB1 mutagenicity is shown in Figure 3.9. The number of revertants using lutein standard and lutein extracts is summarized in Table 3.6, and 3.7.

**Table 3.3** Number of revertants of lutein standard control

Concentration (ug/plate)	Control (without AFB1)
0	251±11
0.02	247±15
0.2	258±10
0.8	261±13
2	243±8
10	264±12

**Table 3.4** Number of revertants of lutein extracts control

Sample	First Dilution	Second Dilution	Third Dilution
A	249±7	254±12	249±9
B	262±13	257±12	243±19
C	248±14	247±11	262±11
D	243±17	258±8	249±10

A: clean corn with ozonation, B: clean corn without ozonation

C: contaminated corn with ozonation, D: contaminated corn without ozonation

**Table 3.5** Concentration of Lutein Extracts (ug/plate)

Sample	First Dilution	Second Dilution	Third Dilution
A	5.70	1.14	0.23
B	4.50	0.90	0.18
C	2.30	0.46	0.092
D	3.2	0.64	0.128

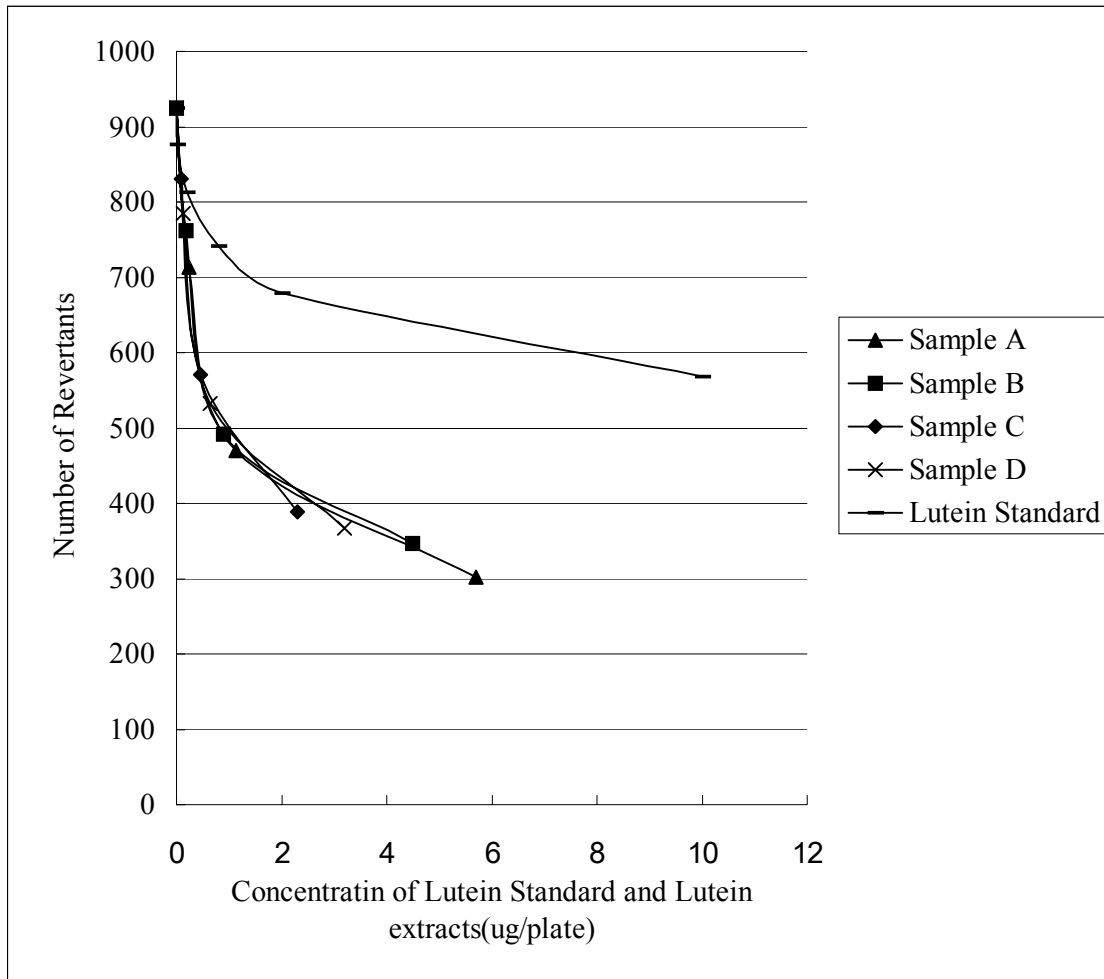
A: clean corn with ozonation, B: clean corn without ozonation

C: contaminated corn with ozonation, D: contaminated corn without ozonation

Lutein standard and lutein extracts inhibited AFB1 (500 ng/plate) mutagenicity in a dose-response manner. Lutein extracts were more efficient than that of lutein standard, as shown in Figure 10. Lutein extracted from ozone treated clean corn was the most efficient, followed by lutein from untreated clean corn, lutein from untreated contaminated corn, and lutein from ozone-treated contaminated corn, since the amount of lutein varied in the different corn samples. This is a further indication that ozone released more lutein in the clean corn and destroyed lutein in the contaminated corn. At the concentration of 0.2 ug lutein standard /plate, the inhibition was 12.1%, while at the similar concentrations of lutein extracts which were a little lower than that of standard, the inhibitions of A, B, C and D were 22.9%, 17.6%, 10.3% and 15.1% respectively. Gonzalez de Mejia et al. (1997) reported that the xanthophylls plus (lutein extracts) was the most efficient. In her research, at the concentration of 0.002 equivalent ug lutein/plate, the inhibition was 11 and 55% for purified lutein and lutein extracts. The result suggests that the lutein extracts have a mixture of antimutagenic agents, and those should have a synergistic effect against AFB1 mutagenicity. Statistical analysis showed that the number of revertants of lutein standard was significantly higher than that of lutein extract with  $P \leq 0.001$ . However, the number of

revertants among lutein extracts was not significantly different with  $P \leq 0.1$ . Lutein extracts from different corn samples had a similar antimutagenic potential (Figure 3.9).

Ozone did not affect the antimutagenic potential of lutein but did affect levels in corn.



**Figure 3.9** Antimutagenic effect of lutein Standard and lutein extracts against AFB1 (500 ng/g) (A: clean corn with ozonation, B: clean corn without ozonation, C: contaminated corn with ozonation, D: contaminated corn without ozonation)

**Table 3.6** Antimutagenic Potency of Lutein Standard Against AFB1 (500 ng/plate) in TA100

Concentration (ug/plate)	Number of Revertants	Percent of Inhibition (%)
0	925±23	0.0
0.02	876±34	5.3
0.2	813±45	12.1
0.8	741±25	19.9
2	679±39	26.6
10	568±50	38.6

**Table 3.7** Antimutagenic Potency Number of Revertants of Lutein Extracts Against AFB1 (500 ng/plate) in TA100

Sample	First Dilution/Percent Inhibition (%)	Second Dilution/Percent Inhibition (%)	Third Dilution/Percent Inhibition (%)
A	302±13/67.4	470±7/49.2	713±12/22.9
B	346±20/62.6	492±4/46.8	762±21/17.6
C	389±10/57.9	571±14/38.3	830±9/10.3
D	367±11/60.3	532±20/42.5	785±13/15.1

A: clean corn with ozonation, B: clean corn without ozonation

C: contaminated corn with ozonation, D: contaminated corn without ozonation

**Table 3.8** Percent Inhibition (%) of Lutein Extracts Against AFB1 (500 ng/plate) in TA100

Sample	First Dilution	Second Dilution	Third Dilution/
A	67.4	49.2	22.9
B	62.6	46.8	17.6
C	57.9	38.3	10.3
D	60.3	42.5	15.1

A: clean corn with ozonation, B: clean corn without ozonation

C: contaminated corn with ozonation, D: contaminated corn without ozonation

Some studies were done on the mechanism of lutein against AFB1 mutagenicity.

Gonzalez de Mejia et al. (1997) reported that in a study on the effect of lutein on the DNA-repair system of Tester strain YG 1024 which is a derivative of TA98, was tested in a preincubation test, a modest inhibition was observed (31% at 10ug lutein/plate) on AFB1 mutagenicity. According to their data, lutein may have some effect on the DNA-repair system of YG 1024. However, when the bacteria were incubated with lutein and S9 first, the AFB1 mutagenicity was inhibited in a dose-response. The percent inhibition of 10 ug lutein/plate was 71%, which is higher than that in the preincubation. In spectrophotometric studies, a new absorption peak was detected at 378 nm when lutein and AFB1 were incubated together (Gonzalez de Mejia et al., 1997). The result indicated that lutein can inhibit AFB1 mutagenicity by forming a complex between lutein and AFB1, therefore limiting the bioavailability of AFB1. In studies conducted by Cardador et al. (2002), it was observed that the greatest inhibitory effect of phenolic compound



present in beans occurred when the phenolic extract (PE) was incubated with AFB1, independent of the first or second incubation in a two-stage incubation protocol. This suggests that PE could be interacting directly and non-enzymatically with the proximate and/or ultimate mutagen (AFB1 8, 9 – epoxide), or forming a complex between the phenolic compounds and AFB1, thereby reducing the bioavailability of AFB1. Mechanistic studies suggest that chlorophyllin can act as an “interceptor molecule” through the formation of tight molecular complexes with carcinogens such as AFB1 (Breinholt et al, 1995). Thus, chlorophyllin may diminish the bioavailability of AFB1.

The mechanism of lutein against AFB1 mutagenicity is most probable the result of a combination of the following reasons: a) lutein may interact directly and non-enzymically with the proximate and/or ultimate mutagen(s); b) formation of a complex between lutein and AFB1; and c) lutein may also affect the metabolic activation of AFB1 by S9 and the expression of AFB1 modified *Salmonella* DNA (Gonzalez de Mejia et al., 1997).

## CHAPTER 4. SUMMARY AND CONCLUSIONS

Aflatoxins in food and feeds cause serious problems in human and animal health. Aflatoxin B1, the most potent of four naturally occurring aflatoxins, is both hepatotoxic and carcinogenic. Under certain conditions, prevention is not always possible although it is the most practical and rational approach. If contamination is already present, treatment must be done to reduce the risk associated with the aflatoxin contaminated food and feeds.

Among all the decontamination methods, ozonation has been shown to be effective in degrading aflatoxin, in contaminated commodities, especially corn, peanuts and cottonseeds. However, limited studies have been done on the suitability and acceptability of the ozonation process especially the influence of ozone on other valuable compounds. The primary objective of this study was to determine the levels of lutein and protein before and after ozonation. It also aimed to determine the antimutegenic potential of lutein extracts using the *Salmonella*/ microsomal mutagenicity assay.

The first study focused on the determination of level of lutein in the ozone-treated corn. Results of the study indicated that the ozone process did change the level of lutein in the corn. In clean corn, the content of lutein in treated corn was higher than that of lutein in untreated corn. On the contrary, the ozone process decreased the lutein content in the contaminated corn. Protein analysis study showed that ozone could destroy protein, which in turn may affect the nutritional quality of the corn.

Lutein standard and lutein extracts showed no mutagenic potential when tested against *Salmonella typhimurium* tester strain TA100. The lutein extracts from corn had a stronger effect on the mutagenicity of AFB1 than lutein standard, perhaps due to the fact that the lutein extracts have a mixture of antimutagenicity agents, and those should have a synergistic effect on AFB1 mutagenicity. Lutein extracts from different corn samples had similar antimutagenic potential. Ozone can not affect the antimutagenic potential of lutein but can affect the levels of lutein in corn.

In conclusion, results from present studies demonstrate that ozone can destroy some antimutagenic compounds and protein as well as aflatoxins. It will affect the nutritional quality of commodities. It is necessary to do further study on bioavailability of lutein and protein before and after ozonation in the future.

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## **VITA**

Yu Wang was born on October 10, 1980, in Hebei, China. She lived in Shijiazhuang, China, until she graduated from high school. In July 2003 she graduated from Hefei University of Technology with a Bachelor of Engineering degree in food science and engineering. After receiving her bachelor's degree, she began pursuing a graduate degree at Louisiana State University and Agricultural and Mechanical College in the Department of Food Science in spring of 2004. She is a candidate for the degree of Master of Science in food science in August 2005. After graduation, she is going to Rutgers, The State University of New Jersey, to attend a doctoral program in the Department of Food Science.